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by

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RAPD CHARACTERIZATION OF THE POPULATION OF *SPHAEROPSIS SAPINEA* IN THE GREAT PLAINS: RESULTS FROM ANALYSIS OF A PRELIMINARY SAMPLE

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SUMMARY

Sphaeropsis shoot blight and canker, caused by *Sphaeropsis sapinea* (syn. *Diplodia pinea*) occurs on coniferous hosts worldwide and has resulted in considerable damage in Great Lakes and Great Plains regions of the United States. Differences in morphology among isolates from the former region suggested the occurrence of two subgroups, referred to as A and B morphotypes. Although morphological characteristics are not always consistent, analysis of random amplified polymorphic DNA markers (RAPDs) of isolates collected in Michigan, Minnesota, and Wisconsin has confirmed the existence of two distinct populations of the pathogen. Other work in our program has indicated differences in host responses (red and jack pines) to inoculation with isolates of these groups.

Although fundamental research on infection biology and control of the pathogen has been conducted in the Great Plains region, the morphotype(s) and degree of variation within the pathogen population have been unknown. Therefore, RAPDs were obtained for a preliminary sample consisting of 18 *S. sapinea* isolates from Austrian, red, Scots, and ponderosa pines in Nebraska, Oklahoma, and South Dakota. Relationships among these isolates, two A and two B isolates from the Lake States region, and an outgroup were determined using both cluster analyses and parsimony methods. Analyses place all these Great Plains region isolates in the same group with the A morphotype isolates. This result sets the stage for a more extensive examination of the population of the fungus in the Great Plains region, and for more intensive investigation of the degree of pathogen variability in particular epidemic locations. These techniques also may help resolve relationships among other *S. sapinea* isolates and related fungi from their numerous hosts throughout the world.

INTRODUCTION

Sphaeropsis sapinea (Fr.:Fr) Dyko & Sutton in Sutton [syn. *Diplodia pinea* (Desmaz.) J. Kickx fil] is the cause of Sphaeropsis shoot blight and canker of *Cedrus* Trew, *Juniperus* L., *Picea* A. Dietr., *Pseudotsuga* Carriére, and over 30 species of *Pinus* L. (Farr et al 1989, Swart et al 1985). The disease occurs worldwide and has been associated with significant economic damage in exotic pine plantations in New Zealand, Australia, and South Africa (Currie and Toes 1978, Zwolinski et al 1990). It also occurs in the Great Lakes and Great Plains regions of the United States on Austrian pine (*P. nigra* Arnold), Scots pine (*P. sylvestris* L.), ponderosa pine (*P. ponderosa* Dougl.)red pine (*P. resinosa* Ait.), and jack pine (*P. banksiana* Lamb.)(Brookhauser and Peterson 1971, Nicholls and Ostry 1990, Palmer and Nicholls 1985, Peterson 1981, Peterson and Wysong 1968, Renlund 1980, Waterman 1939). Although the disease has been observed in these regions for many years, epidemics that have occurred since the 1970's have prompted several more recent examinations of the biology of *S. sapinea*, including investigation of variability within the pathogen population.

Palmer et al (1987) recognized two groups of isolates within *S. sapinea*, which they designated as type A and type B. Isolates of the A type have been obtained from many coniferous hosts throughout the world, whereas B type isolates have been obtained only from red and jack pines in the northcentral United States (Wang et al 1985). We refer to these groupings as morphotypes because the differentiation is made primarily upon morphological criteria and the taxonomic significance of the groupings is unknown (Hawksworth et al 1983). A morphotype isolates grow more quickly on agar media than B morphotype isolates and produce abundant white to gray-green aerial mycelium. Mycelium of B morphotype isolates is dark gray and closely appressed to the agar surface (Palmer et al 1987). Wang et al (1986) reported that conidia of A morphotype isolates are smooth and slightly larger than the pitted conidia of the B morphotype isolates. However, a high degree of variability in the spore surface was subsequently reported (Swart et al 1993). Although host ranges within the north-central United States overlap, differences in virulence between the morphotypes has been demonstrated (unpublished data, James T. Blodgett, current Ph.D. candidate, Department of Plant Pathology, University of Wisconsin-Madison).

We have recently used a polymerase chain reaction procedure to develop an accurate method to distinguish morphotypes as a prerequisite to further pathological studies. The polymerase chain reaction procedure that detects polymorphisms in DNA, random amplified polymorphic DNAs (RAPDs), has been shown to resolve relationships at the population and species level while avoiding the disadvantages of RFLPs (Welsh and McClelland 1990, Williams et al 1990). RAPD markers have been used to distinguish

fungal races, pathotypes, strains, and isolates (Crowhurst et al 1991, Goodwin and Annis 1991, Guthrie et al 1992, Hammerli et al 1992, Ouellet and Seifert 1993, Strongman and MacKay 1993).

Using RAPDs coupled with cluster and parsimony analysis techniques, we confirmed the existence of two distinct entities within *S. sapinea* in the Great Lakes region of United States (Smith and Stanosz 1995). RAPDs were obtained for 16 A and 16 B morphotype isolates collected in Michigan, Minnesota, and Wisconsin from Austrian pine, eastern white pine, jack pine, red pine, and Scots pine. Cluster and parsimony analyses placed all the A morphotype isolates in one group and all the B morphotype isolates in another group.

Fundamental research on infection biology and control of the pathogen has been conducted in the Great Plains region. However lack of knowledge of the morphotype(s) and degree of variation within the pathogen population which was the subject of that research may limit application of those results to other regions. Therefore, we have performed analyses of RAPDs obtained from a preliminary sample of Great Plains region isolates.

MATERIALS AND METHODS

Fungal isolates. *S. sapinea* isolates used in this study (Table 1) included isolates from the Great Plains region and previously examined A and B morphotype isolates from the Great Lakes region (Smith and Stanosz 1995). Eleven Great Plains region isolates were provided from the culture collection of the Center for Semiarid Agroforestry, Rocky Mountain Forest and Range Experiment Station, USDA Forest Service. Two other isolates from this collection were contaminated or showed abnormal characteristics and were discarded. Seven other isolates were obtained from samples collected in September 1994 at the Horning, Nebraska research farm of University of Nebraska. Each culture originated from a single conidium. Four isolates of *Diplodia mutila* (teleomorph *Botryosphaeria stevensii*) from *Juniperus scopulorum* (Tisserat et al 1988) were used as an outgroup in cluster and parsimony analyses. This species was chosen because traditional taxonomy places it close to *S. sapinea* (Sutton 1980).

Genomic DNA extraction. Isolates were grown in 0.5 ml potato dextrose broth (PDB) in 1.5 ml microfuge tubes for 7 days at room temperature. The cultures were centrifuged at 8,000 x g for 5 min and the PDB was aspirated. The pellets were washed twice with 10 mM Tris - 1 mM EDTA. DNA was extracted by a procedure used for plant tissue (Gilbertson et al 1991). The mycelium was ground in 250 µl of extraction buffer (100 mM Tris, pH 8.0, 50 mM EDTA, 500 mM NaCl, 10 mM β-mercaptoethanol) with a Kontes pestle (Vineland, NJ). Another 250 µl of extraction buffer and 33 µl of 20% sodium dodecyl sulfate were added and the mixture was vortexed thoroughly, then incubated at 65°C for 10 min.

Table 1. Fungal isolates used for RAPDs analysis.

Fungus	Isolate No. UW, USFS	Host	Origin
Sphaeropsis sapinea	94-29, 245	*P. sylvestris*	Univ. Nebraska
	94-30, 248	*P. nigra*	Univ. Nebraska
	92-31, 294	*P. nigra*	Univ. Nebraska
	92-32, 305	*P. ponderosa*	Crawford, NE
	92-33, 306	*P. ponderosa*	Crawford, NE
	92-34, 307	*P.ponderosa*	Crawford, NE
	92-35, 309	*P. ponderosa*	Custer,SD
	92-36, 310	*P. ponderosa*	Custer,SD
	92-38, 319	*P. ponderosa*	Pennington, SD
	92-39, 325	*P. nigra*	Norman, OK
	92-40, 326	*P. nigra*	Norman, OK
	94-58	*P. sylvestris*	Horning farm,NE
	94-62	*P. nigra*	Horning farm,NE
	94-66	*P. sylvestris*	Horning farm,NE
	94-71	*P. ponderosa*	Horning farm,NE
	94-73	*P. ponderosa*	Horning farm,NE
	94-86	*P. resinosa*	Horning farm,NE
	94-88	*P. resinosa*	Horning farm,NE
	92-14[a]	*P. nigra*	Dane Co., WI
	92-19[a]	*P. resinosa*	Wood Co., WI
	92-9[b]	*P. banksiana*	Wood Co., WI
	113[b]	*P. banksiana*	Michigan
Diplodia mutila	94-1	*Juniperus scopulorum*	Republic Co., KS
	94-3	*J. scopulorum*	Riley Co., KS
	94-4	*J. scopulorum*	Reno Co., KS
	94-5	*J. scopulorum*	Sedgwick Co., KS

[a] A morphotype isolate as previously determined
[b] B morphotype isolate as previously determined

One-hundred sixty µl of 5 M potassium acetate was added to the mixture, which was then vortexed briefly and centrifuged at 8,000 x g for 10 min. Four-hundred fifty µl of supernatant was transferred to a new 1.5 ml microfuge tube and 225 µl of isopropanol added. This was vortexed briefly and centrifuged at 8,000 x g for 10 min. The supernatant was aspirated and the DNA pellet washed with 70% ethanol, then dried in a Speed-Vac (Savant Instruments Inc., Farmingdale, NY). The DNA pellet was resuspended in 100 µl of distilled water. DNA concentrations were estimated with a miniflourimeter (Model TKO 100, Hoefer Scientific Instruments, San Francisco) and diluted to 5-10 ng/µl if necessary. The DNA was stored at -20°C.

Amplification conditions. Preliminary tests showed that a variation of the standard DNA amplification using long primers (> 17 nucleotides) increased reproducibility of the banding patterns. DNA amplification took place in two steps, the first allowing low stringency annealing of primers and the second allowing high stringency annealing (Welsh and McClelland 1990). The low stringency reaction took place in a 10 µl reaction mixture consisting of 0.25 U Taq DNA polymerase, 1X buffer, 3.75 mM $MgCl_2$, 0.25 mM each dNTP (all purchased from Promega Corp., Madison, WI), 20 µM primer, and 5-10 ng DNA. Primer sequences were as follows: DS2: 5'-CTGCGACTGAATCTTGCAG; DS3: 5'-GCGAAATGTGTCCTTTGATG; DS4: 5'-GTACCCTCAATCTCGTG; DS5: 5'-TTGAGAAGCTGTAGAAGG; DS9: 5'-GAGATCTATGTTGCACC; DS10: 5'-GGAGGATTTGCTAACTGAG; DS16: 5'GGGGATCCCAGTCATGTACCGTTGATCG; DS19: 5'-CAGGTCAGCACCTTTCCATCC. The reaction mixture was overlaid with mineral oil and the following temperature profile was used in a Perkin-Elmer Thermal Cycler 480 (Norwalk, CT): 2 cycles of 5 min at 94°C, 5 min at 40°C, and 5 min at 72°C. Ten cycles of high stringency annealing followed: 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. After this reaction, 90 µl of solution containing 1 U Taq DNA polymerase, 1X buffer, 1.5 mM $MgCl_2$, and 0.125 mM each dNTP was added to each tube. Amplification with high stringency annealing was continued for 25 cycles. DNA fragments were separated on a 0.7% agarose gel (SeaKem LE, FMC BioProducts, Rockland, ME) with Tris-boric acid-EDTA buffer (TBE) and photographed after staining with ethidium bromide. Each isolate was assayed three times by growing a culture, extracting DNA, amplifying the DNA with each primer, and separating the fragments.

Data analysis. Each isolate was scored for the presence or absence of each amplification product. Only amplification products that were present throughout each of three repetitions for at least one isolate were scored. Previously, Southern blots indicated lack of homology of co-migrating fragments in Lake States region isolates (Smith and Stanosz 1995). Thus, non-homology of a few comigrating fragments was assumed and these were scored as separate fragments.

Relationships among isolates were determined by two methods. The first involved calculation of a simple matching coefficient (S_{sm}) for each pair of isolates. The simple matching coefficient was calculated by the formula described by Sneath and Sokal (1973):

$$S_{sm} = m/(m + u)$$

where m is the number of bands found in common between two isolates and u is the total number of bands unique to each isolate. A dendrogram was constructed after cluster analysis of the similarity coefficient by unweighted pair-group method using arithmetic averages (UPGMA)(Sneath and Sokal 1973). These calculations were performed with the programs SIMQUAL and SAHN of the software package NTSYS-pc version 1.80 (Rholf 1993).

For the second method, the binary data matrix was used to construct cladograms using the computer program Phylogenetic Analysis Using Parsimony (PAUP version 3.1.1) (Swofford 1991). Data were analyzed using Wagner parsimony, which assumes that all characters change from one state to another with equal probability. The heuristic algorithm with branch-swapping (tree bisection and reconnection) was used to generate a set of most parsimonious trees. A 50% majority-rule consensus tree was generated by 100 repetitions of the bootstrap algorithm to determine branch strengths.

RESULTS

Amplification products. The eight RAPD primers produced 44 scorable DNA fragments. Twenty-four fragments were polymorphic between the A morphotype and the B morphotype. Eleven fragments were produced only by outgroup isolates. All primers except DS4 yielded a banding pattern that differentiated the two morphotypes.

Data analyses. Both cluster analysis and parsimony yielded similar results. The simple matching coefficients of similarity indicate that the tested Great Plains region isolates are closely related to each other (>89% similarity) and closely related to the Lake States region A morphotype isolates (> 81% similarity) (Fig. 1). Conversely, these Great Plains region isolates are not closely related to the B morphotype isolates (< 45% similarity).

Similar results were obtained using PAUP. Fifty equally parsimonious trees with length of 63 steps were generated using Wagner parsimony (consistency index = 0.70; retention index =0.87), all with the Great Plains region isolates and Lake States A morphotype isolates in one clade and the B morphotype isolates in a separate clade. Bootstrap analysis with 100 repetitions shows that the A morphotype clade is present in 100% of the trees and the B morphotype clade in 97% of the trees (Fig. 2).

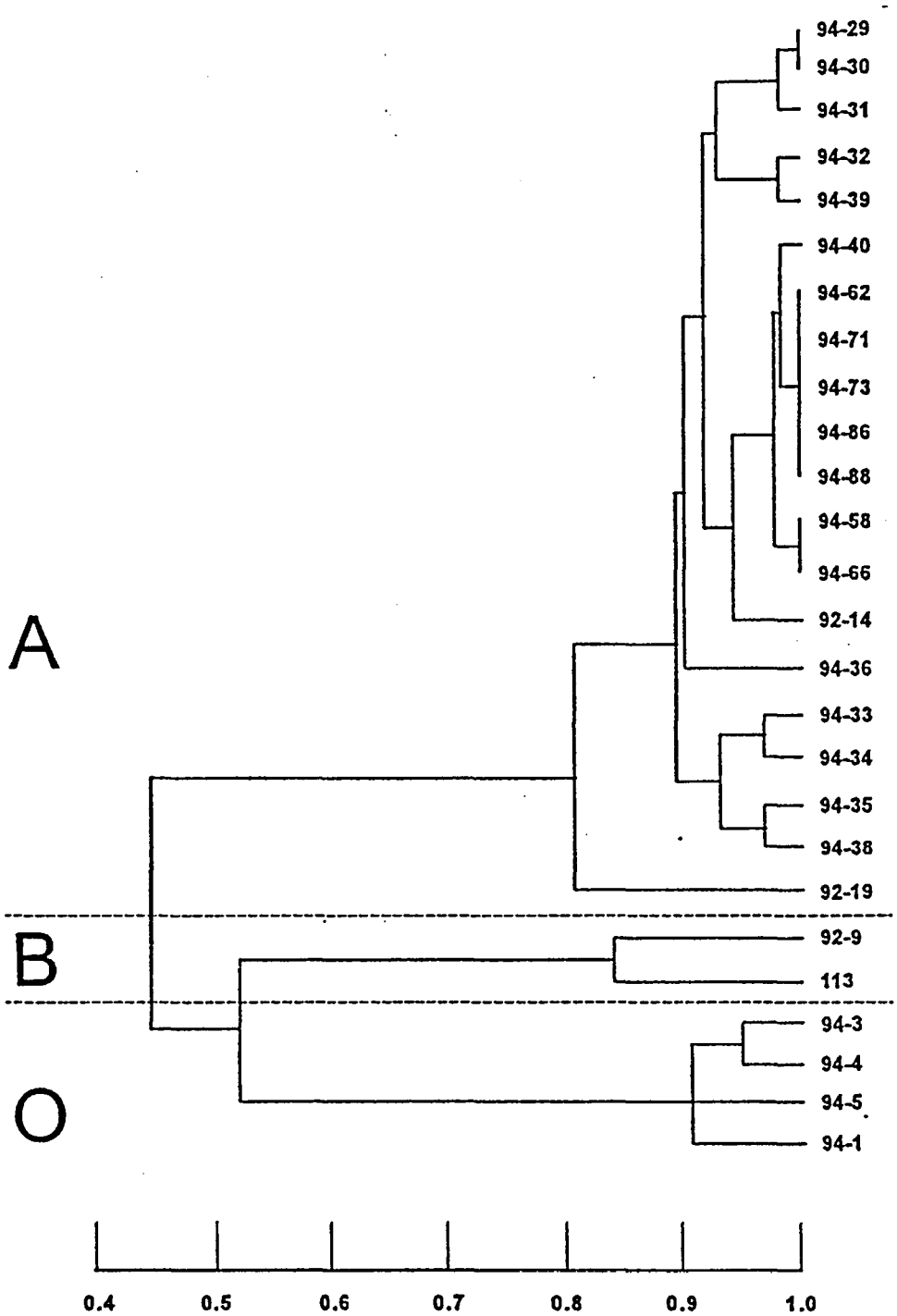

Figure 1. Dendrogram generated by UPGMA using the simple matching coefficient of similarity in the program NTSYS-pc based on 44 RAPD fragments. Dotted lines separate (A) Great Plains region isolates grouped with two *Sphaeropsis sapinea* A morphotype isolates, (B) *S. sapinea* B morphotype isolates, and (O) the outgroup *Diplodia mutila*.

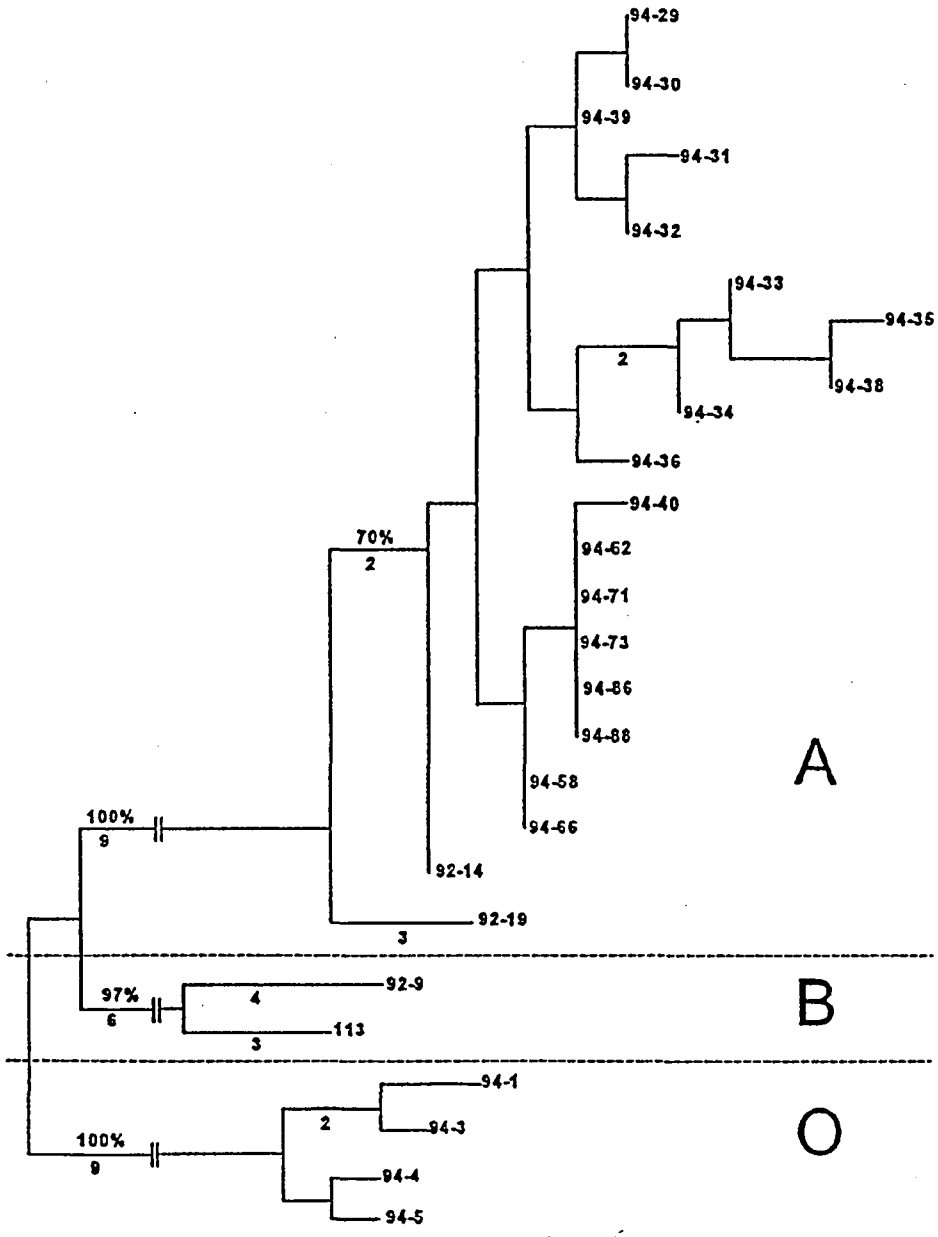

Figure 2. Cladogram of 50% majority-rule consensus of 50 most parsimonious trees showing the relationships of *Sphaeropsis sapinea* and *Diplodia mutila* isolates based on RAPD fragments. Relationships were determined using the heuristic search in PAUP version 3.1.1 (Wagner parsimony). Branch strengths were tested by 100 repetitions of the bootstrap algorithm with branch swapping (number above the line). Branch lengths greater than 1 are given below the line. Dotted lines separate (A) Great Plains region isolates grouped with two *Sphaeropsis sapinea* A morphotype isolates, (B) *S. sapinea* B morphotype isolates, and (O) the outgroup *Diplodia mutila*.

DISCUSSION

Relatively high morphological variability has been reported by many who have examined the fungi currently referred to as *S. sapinea*. Despite reported differences in spore surface characteristics and size, cultural morphology and growth rates, isozymes, and spore germination and germ-tube growth on fungicide-amended medium (Bachi and Peterson 1982, Palmer et al 1987, Swart et al 1991, Swart et al 1993), use of these characters to clearly distinguish groups of isolates within the species has been less than satisfactory. Although we initially used differences in growth rate and cultural morphology to categorize isolates, we also have observed a range in these characteristics between isolates of a particular morphotype and for individual isolates after successive transfers and storage. Swart et al (1991) concluded, after examination of variability in South African isolates, that there may be more than two distinct types or that variation may occur as a continuum without any distinct types or strains.

Results of our previous study indicated, however, that isolates could be differentiated into two very distinct groups using RAPD markers (Smith and Stanosz 1991). Analysis of RAPDs from 18 Great Plains region isolates strengthened this conclusion. All of these isolates grouped with the A morphotype isolates from the Great Lakes region. The two methods of data analysis did not influence this grouping.

The relatively small number of isolates examined in this study probably strongly limits reliability of conclusions that might be drawn. However, grouping of all isolates from the Center for Semiarid Agroforestry collection as the A type strongly suggests this as the morphotype of isolates encountered by Glenn Peterson and used in his research. It might be possible then, to extrapolate characteristics of these isolates to A morphotype isolates from other hosts and regions.

Thus far, B morphotype isolates from the Great Lakes region have been associated with jack pine, or red pine in locations where jack pine also is present. The restriction of Great Plains isolates used to those from Austrian, ponderosa, and Scots pine, and the absence of native jack pine forest in that region may explain the absence of B morphotype isolates in this sample. Obviously, analysis of a broader collection is necessary before the geographic and host range of each morphotype is known.

Although our previous analysis of A and B morphotype isolates from the Great Lakes region did not reveal geographic grouping, some tendency toward geographic grouping appeared within the Great Plains isolates. This might be an artifact of the small sample size. However, it might also indicate relatively little diversity in the population occupying a given location. This could have resulted from a limited number of introductions of this asexual

fungus to the region, or proliferation of only a small number of genotypes during the development of epidemics. Again, examination of a much larger sample of isolates from a broader geographic and host range within the Great Plains region will be necessary to explore these possibilities.

During the September 1994 trip through the region, we obtained many additional isolates from multiple locations in Iowa, Nebraska, and South Dakota. Hosts include several additional species of nursery seedlings, and windbreak, plantation, or ornamental trees. We have also obtained additional isolates from specimens mailed to us from the Black Hills and from North Dakota. As resources become available to support additional analysis, examination of these isolates should yield a more complete understanding of diversity within the *S. sapinea* populations on individual trees, in specific locations, or in the Great Plains as a region. We will then be able to examine relationships with isolates found in other areas in which this pathogen causes serious damage to coniferous trees.

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